

EXHIBIT V
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Simultaneous Determination of α -Fetoprotein and Free β -Human Chorionic Gonadotropin by Element-Tagged Immunoassay with Detection by Inductively Coupled Plasma Mass Spectrometry

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Background: An inductively coupled plasma mass spectrometry (ICP-MS)-based immunoassay has been proposed independently by Baranov et al. (*Anal Chem* 2002;74:1629–36) and our group, but the applicability of this method for multianalyte analysis in clinical samples has not been fully illustrated. We developed a dual-label immunoassay method for the simultaneous determination of α -fetoprotein (AFP) and free β -human chorionic gonadotropin (hCG β) in human serum.

Methods: Monoclonal antibodies immobilized on microtiter plates captured AFP and hCG β , which were detected by use of Eu³⁺-labeled anti-AFP and Sm³⁺-labeled anti-hCG β monoclonal antibodies. Eu³⁺ and Sm³⁺ were dissociated from the immunocomplex with HNO₃ solution (10 mL/L) and delivered by peristaltic pump to the ICP mass spectrometer.

Results: The measurable ranges of AFP and hCG β were 4.6–500 and 5.0–170 μ g/L, respectively, with detection limits of 1.2 and 1.7 μ g/L (3 SD above mean of zero calibrator), respectively. The intraassay imprecision (CV) for AFP was 8.3%, 4.0%, and 2.7% at 16.3, 86, and 354 μ g/L, respectively, and the interassay CV was 10%, 5.7%, and 3.5%. For hCG β , the intraassay CV was 5.4%, 6.4%, and 3.1%, respectively, at 10.5, 45.2, and 105 μ g/L, and the interassay CV was 7.2%, 8.0%, and 3.7%. Comparison with IRMAs for AFP and hCG β yielded correlation coefficients (r^2) of 0.97 and 0.95.

Conclusions: Two proteins can be measured simultaneously by immunoassays using two rare earth elemental tags (Eu³⁺ and Sm³⁺) and ICP-MS detection. The

multielement capability and the multiple potential elemental labels make ICP-MS attractive for multianalyte immunoassays. Implementation of ICP-MS-linked immunoassays may be relatively straightforward because the labeling and immunoreaction procedures have been well developed for clinical time-resolved immunofluorometric assays.

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Simultaneous multianalyte determinations are important for biological studies (1–5). Various multianalyte methods have been developed. Multianalyte immunoassays present several advantages, such as reduced analysis time, minimized repetitions of tedious procedures, and lower cost per test (6). Typically, these multianalyte immunoassay methods involve the use of more than one label. Although several kinds of labels, such as radiolabels or fluorescent, enzyme, or metal ion labels, have been used in the multianalyte immunoassays, the lanthanide chelate labels with time-resolved fluorescence detection are particularly attractive because of high sensitivity and other advantages (7–9). Recently, quantum dots such as CdSe have attracted attention because they can be prepared in a variety of sizes and their fluorescent properties are dependent on the size of the quantum dot (10, 11). Having several quantum dots that all fluoresce at distinct wavelengths under identical excitation offers a dramatic advantage over fluorescence based on organic molecules, but the use of quantum dots in multiplexing are limited by the number of spectrally distinct species that can be prepared. Color-coded microspheres overcome many of the shortcomings of quantum dots because several hundred spectrally different types of beads can be prepared (12). Unfortunately, this labeling system is limited by the number of spectrally distinguished fluorophores, and its use for analyte quantification has encountered difficulties (13). Barcode labels circumvent the problems of fluorophores and quantum dots (14–16). However, the prepa-

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ration of barcode or bio-barcode labels is cumbersome, and the automated quantitative analysis still has some difficulties. In summary, the limitations to the reported approaches in the literatures for multianalyte determination include difficulty in finding a sufficient number of suitable labels that can be prepared easily and can be detected distinctly with similar sensitivities by a single detection technique.

Inductively coupled plasma mass spectrometry (ICP-MS),¹ a powerful technique for the simultaneous determination of multiple elements with minimal pretreatment of samples, offers excellent sensitivity for a wide range of elements. The combination of ICP-MS with immunoassays enables analysis of biological materials (17–23). Baranov et al. (18) described several novel ICP-MS-linked immunoassays that use both nanogold and lanthanide-tagged antibodies. Commonly used immunoaffinity separation techniques have been successfully coupled to ICP-MS to measure the concentrations of target proteins in complex biological samples. Parallel studies carried out in our laboratory used thyroid-stimulating hormone and thyroxine as model compounds to demonstrate the potential of ICP-MS as a detector for the Eu^{3+} that is attached to an immunoreagent (19, 20). We also established a sandwich-type immunoreaction coupled to ICP-MS with colloidal gold nanoparticle-labeled antibody (21). The measurement of multiple proteins simultaneously by use of distinguishable element-tagged antibodies with ICP-MS has been illustrated by Quinn et al. (23), who used colloidal gold and Eu^{3+} as labels. As an excellent element-specific detection technique, the detection limits of ICP-MS for most elements range from parts per billion to parts per trillion, or even lower. Elements such as rare earths and noble metals, as well as some of the transition metals, have the highest sensitivity with detection limits down to 0.01–0.1 ng/L (24). Those elements, their stable isotopes, or the unique combination of them are candidates for labeling bioactive molecules, especially those that occur at naturally low concentrations in the body and environment. However, the value of ICP-MS-based immunoassays in clinical analysis has not been fully understood, and further research in this area is needed for the use of this methodology in practical applications.

α -Fetoprotein (AFP) and β -human chorionic gonadotropin (hCG β) are important tumor markers and also serve as aids in prenatal detection of fetal malformations (25–28). Numerous immunologic methods for determining AFP and hCG β concentrations have been described (29–35), but few methods other than time-resolved immunofluorometric assays and enzyme immunoassays can measure them simultaneously (28, 36, 37). In this report, we demonstrate the simultaneous measurement of AFP

and hCG β in clinical sample with ICP-MS as the end-point detection method. The commonly used elements in the present study, Eu^{3+} and Sm^{3+} , have detection limits down to 0.01 ng/L in ICP-MS with good biocompatibility and state-of-the-art bioconjugation methods. The proposed method shows the potentially powerful capability of ICP-MS-based multianalyte immunoassay for clinical use.

Materials and Methods

REAGENTS AND BUFFERS

AFP, hCG β , mouse monoclonal anti-AFP IgG, and mouse monoclonal anti-hCG β IgG for labeling or immobilization were from Scripps Laboratories. We used purified water (18 M Ω /cm) for all experiments. N' -[*p*-isothiocyanato-benzyl]-diethylene-triamine- N^1, N^2, N^3, N^3 -tetraacetate europium and N' -[*p*-isothiocyanato-benzyl]-diethylene-triamine- N^1, N^2, N^3, N^3 -tetraacetate-samarium were from Tianjing Radio Medical Institute. Microtiter strips were obtained from NUNC Co. Superpurity HNO_3 was obtained from Beijing Chemical Reagents Institute. Other reagents were of analytical grade.

The coating buffer was 100 mmol/L sodium carbonate buffer (pH 9.5) containing 9 g/L NaCl and 0.4 g/L NaN_3 . The blocking buffer was 50 mmol/L Tris-HCl (pH 7.0) containing 9 g/L NaCl, 0.4 g/L NaN_3 , and 10 g/L bovine serum albumin. The assay buffer was 50 mmol/L Tris-HCl (pH 7.8) containing 20 g/L bovine serum albumin, 0.4 g/L NaN_3 , 9 g/L NaCl, and 0.4 mL/L Tween 20. The wash buffer was 50 mmol/L Tris-HCl (pH 7.4) containing 0.4 g/L NaN_3 , 9 g/L NaCl, and 0.4 mL/L Tween 20.

INSTRUMENTATION

We used a Perkin-Elmer/Sciex Elan 6000 ICP mass spectrometer fitted with a cross-flow nebulizer. The instrument settings were optimized daily. Typically, nebulizer gas flow was 1.02 L/min, lens voltage was 8 V, and radio frequency (RF) power was 1150 W. Peak hopping mode was used in the experiment. The chromatographic separation system included a Model EP-1 Econo Pump and a Model EM-1 Econo UV monitor (Bio-Rad).

ANTIBODY LABELING

The anti-AFP and anti-hCG β monoclonal antibodies were labeled with Eu^{3+} and Sm^{3+} chelates, respectively. Briefly, 1 g/L antibody in 50 mmol/L sodium bicarbonate buffer (pH 9.5) was reacted with 0.35 mg of Eu^{3+} or Sm^{3+} chelate labeling reagent and incubated at 4 °C overnight. The Eu^{3+} - or Sm^{3+} -chelate-labeled antibody was purified on a Sephadex G-25 column (1.5 \times 25 cm) and eluted with 50 mmol/L Tris-HCl buffer (pH 7.0) containing 9 g/L NaCl and 0.5 g/L NaN_3 . The unlabeled Eu^{3+} concentration was determined by comparison with known Eu^{3+} standards. The concentration of Eu^{3+} -labeled anti-AFP monoclonal antibody was calculated as:

¹ Nonstandard abbreviations: ICP-MS, inductively coupled plasma mass spectrometry; AFP, α -fetoprotein; hCG β , β -human chorionic gonadotropin; and RF, radio frequency.

$$c = \frac{A_{280} - 0.008c_{Eu^{3+}}}{1.34}$$

where A_{280} is the absorbance of the Eu^{3+} -labeled anti-AFP monoclonal antibody at 280 nm, 1.34 is the absorbance of 1 g/L Eu^{3+} -labeled anti-AFP monoclonal antibody at 280 nm, and 0.008 is the absorbance of 1 μ mol/L Eu^{3+} chelate at 280 nm. The conjugation yield was obtained by calculating the ratio of the concentration of Eu^{3+} -labeled anti-AFP monoclonal antibody to the Eu^{3+} concentration in the solution. The conjugation yield of Sm^{3+} -labeled anti-hCG β monoclonal antibody was calculated similarly. One mole of anti-AFP monoclonal antibody was labeled with 7.1 moles of Eu^{3+} chelate, whereas 1 mole of anti-hCG β monoclonal antibody was labeled with 12.3 moles of Sm^{3+} chelate.

COATING OF MICROTITRATION WELLS

To immobilize the anti-AFP and anti-hCG β monoclonal antibodies on the well surfaces, we added 200 μ L of each to the wells at a concentration of 5 mg/L and allowed them to adsorb. The microtiter strips were washed twice with the wash solution, 300 μ L of blocking buffer was added to each well, and the strips were incubated overnight at 4 °C. After removal of the blocking buffer, the strips were stored at 4 °C in sealed bags until use.

ASSAY PROTOCOL

The typical sandwich format of a noncompetitive immunometric assay was used. The coated microtiter strip wells were washed twice with assay buffer, 25 μ L of calibrator or serum was pipetted into the well, and then 200 μ L of lanthanide-labeled anti-AFP and anti-hCG β antibodies (1 mg/L) was added to each microtiter well. Subsequently, the microtiter strip wells were incubated with continuous shaking at room temperature and then washed six times with wash buffer to separate the unreacted reagent from the microtiter strip well. We then added 200- μ L aliquots of HNO_3 solution (10 mL/L) to each well to dissociate the Eu^{3+} and Sm^{3+} ions from the labeled monoclonal antibodies. After 1 min of continuous shaking of the microtiter strips (40 rpm) at room temperature in the TS-1 shaking apparatus (Jiangsu), the solutions were introduced into the ICP-MS instrument by a peristaltic pump at a flow rate of 1 mL/min. The ^{153}Eu and ^{152}Sm intensities by ICP-MS were proportional to the concentrations of AFP and hCG β in the sample, respectively.

Two determinations were performed for each sample. Analytical results were obtained by calculating the mean values of two determinations of each sample. The correlation was calculated with a linear least-squares method, and agreement was tested by the Student *t*-test.

SAMPLE COLLECTION

Blood samples were collected from 20 patients at the Third Hospital of Peking University (Beijing, China), after the patients gave informed consent. After clotting, the

serum and packed cells were separated by centrifugation at 1000g. The serum samples were then stored at -20 °C under nitrogen gas until they were assayed.

IRMA PROCEDURES

Serum AFP and hCG β were measured by the use of IRMAs (Beijing Chemclin Biotech Co., Ltd) according to the manufacturer's instructions. Briefly, 50 μ L of calibrator or serum sample was pipetted into a coated tube, after which 50 μ L of ^{125}I -labeled AFP or hCG β antibody was added to each coated tube. The microtiter strip wells were incubated for 2 h with continuous shaking at 37 °C and then washed five times with water to separate the unreacted reagent from the coated tubes. The radioactivity of the coated tube was measured to calculate the analyte concentration.

Results

OPTIMIZATION OF INSTRUMENT SETTINGS

The isotopes of highest abundance for both europium and samarium (^{153}Eu and ^{152}Sm) were chosen for this experiment. We used Eu^{3+} and Sm^{3+} standard solutions (5 μ g/L) to optimize key ICP-MS instrument settings, including RF power, nebulizer argon flow, and lens voltage. The dependence of ^{153}Eu and ^{152}Sm signal intensities on the argon nebulizer gas flow was studied at flow rates of 0.7–1.1 L/min. ^{153}Eu and ^{152}Sm behaved similarly, with a common peak at ~ 1 L/min (Fig. 1). The maximum intensity of ^{153}Eu was twice that of ^{152}Sm , reflecting the higher abundance of ^{153}Eu (52.2% for ^{153}Eu and 26.7% for ^{152}Sm). The chosen nebulizer gas flow (argon) for both ^{153}Eu and ^{152}Sm was 1.02 L/min for these studies. The RF power and lens voltage used in this experiment were also optimized; the optimum values were 1150 W and 8 V, respectively.

ASSAY CHARACTERISTICS

Calibration curve and detection limit. A typical calibration curve is shown in Fig. 2. The measurable range for AFP

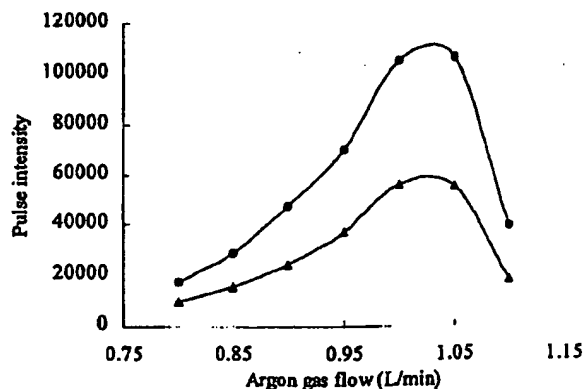


Fig. 1. Optimization of argon gas flow in ICP-MS. \bullet , ^{153}Eu ; Δ , ^{152}Sm . Sm^{3+} and Eu^{3+} concentration, 5 μ g/L; RF, 1250 W; lens voltage, 8 V.

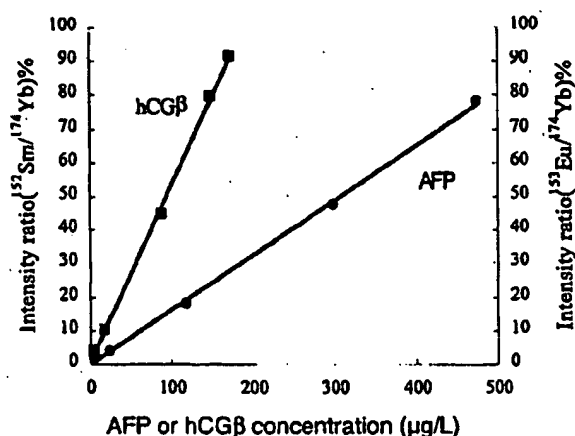


Fig. 2. Calibration curves for AFP (●) and hCGβ (■).

was 4.6–500 μg/L, and that for hCGβ was 5.0–170 μg/L. The regression equations for the calibration curves were: $I = 0.16c + 0.06$ for AFP ($r^2 = 0.9984$) and $I = 0.52c + 0.96$ for hCGβ ($R^2 = 0.9987$), where I is the relative intensity of the ICP-MS signal, and c is the concentration. The lower limits of detection, defined as the concentrations corresponding to a signal 3 SD above the mean of 12 replicates of the zero calibrator, were 1.2 μg/L for AFP and 1.7 μg/L for hCGβ.

Imprecision. The intraassay imprecision (CVs) were ~3–8% for AFP and ~3–5% for hCGβ (Table 1). The between-assay CVs for duplicate analyses over 6 days were 3.5–5.6% for AFP and 3.7–7.2% for hCGβ (Table 1).

Recovery. The recoveries of AFP (16.5, 84.6, and 213 μg/L) and hCGβ (6.8, 52.4, and 108 μg/L) added to samples (Table 2) were 90–104% and 88–110%, respectively.

Linearity. Three serum samples with high AFP and hCGβ concentrations were diluted with AFP- and hCGβ-free human serum. Measured concentrations were 90.8–119% of expected concentrations (Table 3).

Comparison with IRMA. Comparisons with IRMAs for AFP and hCGβ are shown in Figs. 3 and 4, respectively.

Table 1. Within- and between-assay imprecision for AFP and hCGβ.

Sample	Concentration, μg/L	Relative SD, %	
		Within-assay	Between-assay
AFP	16.3	8.3	9.6
	86.2	4.0	5.7
	354.0	2.7	3.5
hCGβ	10.5	5.4	7.2
	45.2	6.4	8.0
	105.0	3.1	3.7

* $n = 6$. Between-assay study used means of daily duplicates.

Table 2. Recoveries for AFP and hCGβ by the proposed ICP-MS-linked immunoassay.

Sample	Original, μg/L	Added, μg/L	Measured, μg/L	Recovery, %
AFP	16.5	48.0	66.5	104
	84.6	48.0	128	90
	213	48.0	260	98
hCGβ	6.8	25.0	28.9	88
	52.4	25.0	80	110
	108	25.0	132	96

The regression equations were: $y = 0.86x + 6.9$ μg/L ($r^2 = 0.97$) for AFP and $y = 1.00x - 0.64$ μg/L ($r^2 = 0.95$) for hCGβ. The standard deviation of the residuals was 4.1 μg/L for AFP ($n = 20$) and 3.3 μg/L for hCGβ ($n = 20$), respectively. The mean results were not significantly different (Student *t*-test).

STABILITIES OF LANTHANIDE-LABELED MONOCLONAL ANTIBODIES

We studied the stabilities of lanthanide-labeled mouse monoclonal anti-AFP IgG and mouse monoclonal anti-hCGβ IgG by storing each antibody (at 50 mg/L) at 4 °C for 1 week, 4 weeks, and 3 months before they were used in the assay. The calibration curves obtained were compared with the calibration curve obtained before the start of the storage experiment. No significant decrease in activity was observed.

Discussion

The most striking features of ICP-MS are its superior sensitivity for a wide range of elements and its ability to determine the isotope composition of a sample with pretreatment procedures that are less cumbersome than those for other MS techniques. However, the application of ICP-MS to biological fluid and tissue samples has been limited to the investigation of elements present in ultra-trace amounts (38, 39). ICP-MS analyses of biological samples need not be limited to analytes that include metal elements. The recent development of metal-tagged antibodies, which are designed for time-resolved immunofluorometric assays, makes possible the use of ICP-MS in immunoassays. Antigens of interest are reacted with their complementary metal-tagged antibodies. After separation

Table 3. Linearity as tested by dilutions of samples with AFP- and hCGβ-free human serum.

Sample	Original concentration, μg/L	Percentage of expected concentration				
		2-fold dilution	4-fold dilution	8-fold dilution	16-fold dilution	32-fold dilution
AFP	43.5	95	106	94	110	
	84.6	94	106	103	91	
	213	95	100	97	101	92
hCGβ	36.8	97	102	109		
	75.1	104	95	109	96	
	134	104	103	95	108	119

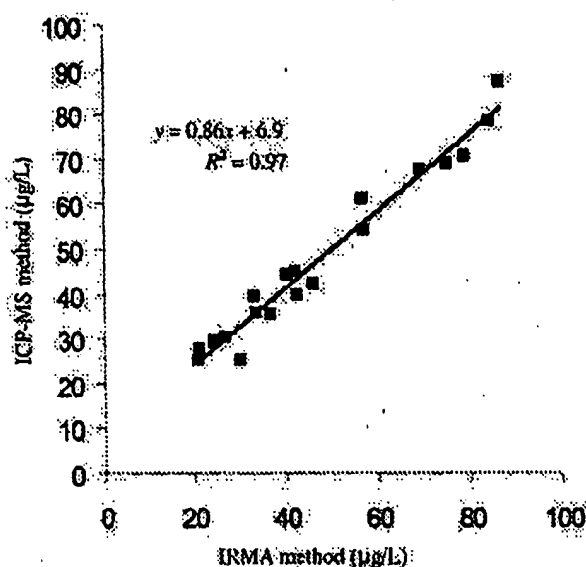


Fig. 3. Relationship between the proposed method and IRMA for AFP in human serum.

from nonreacting proteins, the elemental tags conjugated to the antibodies are measured by ICP-MS to determine the antigen concentrations in the samples (18–20). Because no special fluorescent properties are needed, the potential elemental labels for ICP-MS-linked immunoassay are not limited to the four lanthanides (Eu^{3+} , Sm^{3+} , Tb^{3+} , and Dy^{3+}).

The labels for ICP-MS-linked immunoassay should possess the properties of (a) having a highly sensitive response in ICP-MS and being free from spectral interference; (b) occurring at naturally low concentrations in the

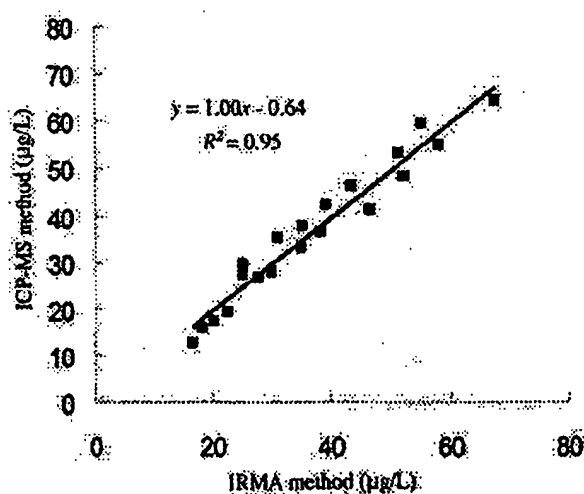


Fig. 4. Relationship between the proposed method and the IRMA for hCG β in human serum.

body and environment; and (c) having good biocompatibility and the ability to easily conjugate to biomolecules. On the basis of the above criteria, rare earth elements, such as europium, samarium, terbium, and dysprosium, can be considered ideal labels for ICP-MS-linked immunoassay because these elements offer good biocompatibility and are easily conjugated to biomolecules. Another advantage of the use of these elements as labels is that they offer high sensitivity for trace species in biological samples, which can satisfy the requirement of routine clinical analysis. In addition, a low background can be expected because these elements hardly exist in biological fluids or tissues. Those properties have been well demonstrated by many reports on time-resolved fluorescent immunoassays (7–9).

The other rare earth elements, including lutetium, yttrium, lanthanum, cesium, praseodymium, neodymium, gadolinium, holmium, erbium, thulium, ytterbium, and scandium, would also be potentially applied to ICP-MS-linked immunoassays because they could satisfy the first and second criteria mentioned above. Methods for their bioconjugation, however, have not been developed to the best of our knowledge. Noble metals, such as gold and ruthenium, would also be candidates for labels because ICP-MS can measure them at very low concentrations and they have been used in immunoassays with spectrometric and electrochemical detection. The other noble metals, such as silver, could also be determined sensitively by ICP-MS, but their biocompatibility is not satisfactory when conjugated to proteins. Some of the transition metals, such as iron, cobalt, and copper, can be measured with high sensitivity by ICP-MS, but these elements may also exist in biological fluids to some extent. Spectral interference may also be a problem, especially for the determination of iron-labeled biomolecules.

Quinn et al. (23) have discussed the simultaneous determination of two proteins with ICP-MS using nanogold clusters and Eu^{3+} as labels. One of the disadvantages of using nanogold clusters in this method is that gold has a high affinity for the surfaces of a typical ICP-MS sample introduction system, as pointed out by the authors. Another disadvantage is that the responses of nanogold clusters and Eu^{3+} in ICP-MS are not consistent, and two internal standards, iridium and holmium, must be used. Because lanthanides are a group of closely related elements having similar physical and chemical properties, a single or at most two internal standards are sufficient to control for instrumental drift for the determination of all of the lanthanides. Eu^{3+} and Sm^{3+} are used in the present study because, among all the elements in the periodic table, lanthanides are the most sensitively detected by ICP-MS, with detection limits down to 0.01 ng/L, and because they occur at very low concentrations in body fluids and the environment.

The detection limits of time-resolved immunofluoro-

metric assays for the simultaneous determination of AFP and hCG β with lanthanide chelates are 0.025 $\mu\text{g/L}$ for AFP and $\leq 0.2 \mu\text{g/L}$ for hCG β (36). The detection limits of the lanthanide-labeled ICP-MS-linked immunoassay were 1.2 $\mu\text{g/L}$ for AFP and 1.7 $\mu\text{g/L}$ for hCG β . The ratio of labeled lanthanide to corresponding antibody is an important factor affecting the sensitivity of detection of the ICP-MS-linked immunoassay because the ICP-MS signal intensity is linearly proportional to the number of atoms of the tags. It is possible that the detection limit of the present method can be improved by multiple labeling. In practice, however, improving the detection limit of an immunoassay by increasing the number of labels attached to an antibody has been limited because of the interference of the bulky labels in antibody-antigen binding and the increased fraction of nonspecific binding. In the present study, 1 mole of anti-AFP monoclonal antibody was labeled with 7.1 moles of Eu^{3+} , whereas the anti-hCG β monoclonal antibody was labeled with 12.3 moles of Sm^{3+} . As indicated in the *Results*, the maximum intensity of ^{153}Eu is twice that of ^{152}Sm at the same concentration because the natural abundance of ^{153}Eu is approximately twice that of ^{152}Sm , and we used those ratios only for the convenience of data processing. The nanoparticle-antibody bioconjugates provide a novel solution for this problem without compromising the binding affinity.

When measuring the element tags by MS, the signal enhancement is proportional to the number of atoms of the tag isotope. Because nanoparticles have significant numbers of atoms per conjugate, the increase in sensitivity is obvious. In a previous work, we developed an immunoassay by coupling a sandwich-type immunoreaction to ICP-MS with colloidal gold nanoparticles as labels on goat anti-rabbit IgG (21). In their study, another group showed that different immunoassay methods, such as centrifugal filtration, protein A affinity, and size-exclusion gel filtration, can be linked with ICP-MS immunoassays using nanogold cluster labels (18). However, the use of colloidal gold or nanogold clusters as labels is limited by constraints of specificity and immunoreactivity, and continuing efforts to alleviate the nonspecific binding are required for further improvements in sensitivity.

An alternative method is to use lanthanide-dye-conjugated polystyrene nanoparticles as labels; these types of nanoparticles were used recently in a time-resolved immunofluorometric assay and had the advantages of enhanced monovalent binding affinity and a high specific activity for the nanoparticle-antibody bioconjugate (40, 41). The nanoparticle, 107 nm in diameter, contained $>30\,000$ europium atoms and could be used in ICP-MS-linked immunoassays to improve the detection limit without the problems of diminished activity and specificity. It can therefore be inferred that element-labeled ICP-MS immunoassays can provide sensitivities comparable to those for time-resolved immunofluorometric assays with the potential for use in multianalyte determinations incorporating large numbers of tags.

ICP-MS-linked immunoassays have many advantages over conventional time-resolved immunofluorometric assays (18). ICP-MS-linked immunoassays do not require addition of enhancement solution, as time-resolved immunofluorometric assays do, because the lanthanides themselves are detected directly. The dissociation of lanthanides from antigen-antibody complexes by acidification enables long-term storage before analysis, simplifying assay protocols. The inherent ability of ICP-MS to remain free from interference by organic or inorganic species makes the operational procedure easier. The immunoassay steps are the same as for time-resolved fluorescence except for the detection step. After immunoreaction, enhancement solution is added to the microtiter strip well, which is shaken for 5 min to allow the formation of fluorescent complex for time-resolved fluorescence detection, whereas HNO_3 solution (10 mL/L) is added to dissociate Eu^{3+} and Sm^{3+} ions from the labeled monoclonal antibodies for ICP-MS detection. Because the immunoreaction steps of the two methods are identical, their analytical throughput is the key for comparing their analytical ability. The throughput of time-resolved fluorescence is 1–2 s/sample with automated instruments. In the present study, the throughput was ~ 1 min/sample for two elements. One of the reasons for the lower throughput of the ICP-MS detection immunoassay is that the sampling system was not designed specifically for immunoassays. The advantage of ICP-MS is its ability to analyze multiple analytes, not its analysis speed, compared with time-resolved fluorescence. Because the antibody preparation and immunoreaction steps in the ICP-MS-linked immunoassay are the same as those in time-resolved immunofluorometric assays, which are familiar to clinical analysts, this method can be easily accepted and used by clinical chemists.

One of the promising applications of element-labeled immunoassay combined with ICP-MS detection is in the development of SI-traceable primary methods for interlaboratory comparisons and quality assurance by so-called isotope dilution. Isotope dilution is a calibration technique based on altering the natural relative abundance of two stable isotopes by adding a known amount of an enriched isotope of the analyte to the sample (42). Measurement of this altered ratio allows very accurate quantification because the added enriched isotope acts analogously to an ideal internal standard. The uncertainty of the exact isotopic ratio, the amount of the enriched analyte added, and the weight or volume of the sample are the only sources of error that can affect the results. Therefore, isotope-dilution analysis provides a definitive technique and absolute quantification with excellent precision and accuracy compared with other analytical methods. The possibility of using an isotope dilution in the present method has been under investigation in our laboratory.

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References

- MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science* 2000;289:1760–3.
- Chan WCW, Maxwell DJ, Gao XH, Bailey RE, Han MY, Nie SM. Luminescent quantum dots for multiplexed biological detection and imaging. *Curr Opin Biotechnol* 2002;13:40–6.
- Moreno-Bondi MC, Alarie JP, Vo-Dinh T. Multi-analyte analysis system using an antibody-based biochip. *Anal Bioanal Chem* 2003;375:120–4.
- Rowe CA, Scruggs SB, Feldstein MJ, Golden JP, Ligler FS. Array biosensor for simultaneous identification of bacterial, viral, and protein analytes. *Anal Chem* 1999;71:433–9.
- Delehanty JB, Ligler FS. A microarray-immunoassay for simultaneous detection of proteins and bacteria. *Anal Chem* 2002;74:5681–7.
- Brecht A, Abuknesha R. Multianalyte immunoassays application to environmental analysis. *Trac-Trend Anal Chem* 1995;14:361–71.
- Wu FB, Han SQ, Xu T, He YF. Sensitive time-resolved fluorimetric assay for simultaneous detection of serum thyroid-stimulating hormone and total thyroxine with Eu and Sm as labels. *Anal Biochem* 2003;314:87–96.
- Bookout JT, Joaquim TR, Magin KM, Rogan GJ, Lirette RP. Development of a dual-label time-resolved fluorometric immunoassay for the simultaneous detection of two recombinant proteins in potato. *J Agric Food Chem* 2000;48:5868–73.
- Eriksson S, Vehnäinen M, Jansen T, Meretoja V, Saviranta P, Pettersson K, et al. Dual-label time-resolved immunofluorometric assay of free and total prostate-specific antigen based on recombinant Fab fragments. *Clin Chem* 2000;46:658–68.
- Bruchez M Jr, Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor nanocrystals as fluorescent biological labels. *Science* 1998;281:2013–5.
- Chan WCW, Nie SM. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. *Science* 1998;281:2016–8.
- Han MY, Gao X, Su JZ, Nie SM. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat Biotechnol* 2001;19:631–5.
- Reiss BD, Freeman RG, Walton ID, Norton SM, Smith PC, Stonas WG, et al. Electrochemical synthesis and optical readout of striped metal rods with submicron features. *J Electroanal Chem* 2002;522:95–103.
- Nicewarner-Pena SR, Freeman RG, Reiss BD, He L, Pena DJ, Walton ID, et al. Submicrometer metallic barcodes. *Science* 2001;294:137–41.
- Walton ID, Norton SM, Balasingham A, He L, Oviso DF, Gupta D, et al. Particles for multiplexed analysis in solution: Detection and identification of striped metallic particles using optical microscopy. *Anal Chem* 2002;74:2240–7.
- Nam JM, Thaxton CS, Mirkin CA. Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science* 2003;301:1884–6.
- Deng AP, Liu HT, Jiang SJ, Huang HJ, Ong CW. Reaction cell inductively coupled plasma mass spectrometry-based immunoassay using ferrocene tethered hydroxysuccinimide ester as label for the determination of 2,4-dichlorophenoxyacetic acid. *Anal Chim Acta* 2002;472:55–61.
- Baranov VI, Quinn Z, Bandura DR, Tanner SD. A sensitive and quantitative element-tagged immunoassay with ICPMS detection. *Anal Chem* 2002;74:1629–36.
- Zhang C, Wu FB, Zhang XR. ICP-MS-based competitive immunoassay for the determination of total thyroxine in human serum. *J Anal At Spectrom* 2002;17:1304–7.
- Zhang C, Wu FB, Zhang YY, Wang X, Zhang XR. A novel combination of immunoreaction and ICP-MS as a hyphenated technique for the determination of thyroid-stimulating hormone (TSH) in human serum. *J Anal At Spectrom* 2001;16:1393–6.
- Zhang C, Zhang ZY, Yu BB, Shi JJ, Zhang XR. Application of the biological conjugate between antibody and colloid Au nanoparticles as analyte to inductively coupled plasma mass spectrometry. *Anal Chem* 2002;74:96–9.
- Baranov VI, Quinn Z, Bandura DR, Tanner SD. The potential for elemental analysis in biotechnology. *J Anal At Spectrom* 2002;17:1148–52.
- Quinn ZA, Baranov VI, Tanner SD, Wraga JL. Simultaneous determination of proteins using an element-tagged immunoassay coupled with ICP-MS detection. *J Anal At Spectrom* 2002;17:892–6.
- Horlick G, Montaser A. Analytical characteristics of ICPMS. In: Montaser A, ed. *Inductively coupled plasma-mass spectrometry*. New York: Wiley-VCH, 1998:506–7.
- Berry E, Aitken DA, Crossley JA, Macri JN, Connor JM. Analysis of maternal serum α -fetoprotein and free β -human chorionic gonadotropin in the first trimester—implications for down-syndrome screening. *Prenat Diagn* 1995;15:555–65.
- Gregory JJ, Finlay JL. α -Fetoprotein and β -human chorionic gonadotropin—their clinical significance as tumour markers. *Drugs* 1999;57:463–7.
- Norgaardpedersen B, Alfthan H, Arends J, Hogdall CK, Larsen SO, Stenman UH, et al. A new simple and rapid dual assay for α -AFP and free β -hCG in screening for down-syndrome. *Clin Genet* 1994;45:1–4.
- Ito K, Oda M, Tsuji A, Maeda M. Simultaneous determination of α -fetoprotein, human chorionic gonadotropin and estradiol in serum of pregnant women by time-resolved fluorimetric assay. *J Pharm Biomed Anal* 1999;20:169–78.
- Perkins S, Collins S, Kenten E, Mckeon K, Mossner E, Saul R. An automated electrochemiluminescence immunoassay for the determination of α -1-fetoprotein (AFP). *Clin Chem* 1995;41:S67–8.
- Chu X, Lin ZH, Shen GL, Yu RQ. Studies on the piezoelectric immunosensor for the determination of α -fetoprotein (AFP). *Chem J Chin Univ* 1996;17:870–3.
- Hall LO, Haehner B, Swann S, Feliciano JA, Feliciano D, Miller D, et al. Development of a quantitative method for the determination of total β -hCG from whole blood for the Stratus[®] CSSTAT fluorometric analyzer [Abstract]. *Clin Chem* 2000;46(Suppl 6):A11.
- Santandreu M, Alegret S, Fabregas E. Determination of β -HCG using amperometric immunosensors based on a conducting immunocomposite. *Anal Chim Acta* 1999;396:181–8.
- Gunupudi SR, Cheung H, Blem J, Bethell D. Quantitative determination of HCG using Medix Biotech's contrast HCG urine/serum test [Abstract]. *Clin Chem* 1996;42(Suppl 6):S130.
- Mack M, Oed M, Amtmann R, Schlett R. Liaison[®] hCG—an automated chemiluminescent immunoassay for the determination of human chorionic gonadotropin [Abstract]. *Clin Chem* 1997;43(Suppl 6):S174.
- Yamagata Y, Katoh H, Nakamura K, Tanaka T, Satomura S, Matsuura S. Determination of α -fetoprotein concentration based on liquid-phase binding assay using anion exchange chromatography and sulfated peptide introduced antibody. *J Immunol Methods* 1998;212:161–8.
- Pettersson K, Alfthan H, Stenman UH, Turpeinen U, Suonpaa M, Soderholm J, et al. Simultaneous assay of α -fetoprotein and free β subunit of human chorionic gonadotropin by dual-label time-resolved immunofluorometric assay. *Clin Chem* 1993;39:2084–9.
- Macri JN, Spencer K, Anderson R. Dual analyte immunoassay

- new approach to neural tube defect and Down's syndrome screening. *Ann Clin Biochem* 1992;29:390-6.
38. Sanz-Medel A, Montes-Bayon M, Sanchez MLF. Trace element speciation by ICP-MS in large biomolecules and its potential for proteomics. *Anal Bioanal Chem* 2003;377:236-47.
39. Patriarca M. The contribution of inductively coupled plasma mass spectrometry to biomedical research. *Microchem J* 1996;54:262-71.
40. Hama H, Soukka T, Lonnberg S, Paukkunen J, Tarkkinen P, Lovgren T. Zeptomole detection sensitivity of prostate-specific antigen in a rapid microtitre plate assay using time-resolved fluorescence. *Luminescence* 2000;15:351-5.
41. Soukka T, Paukkunen J, Hama H, Lonnberg S, Lindroos H, Lovgren T. Supersensitive time-resolved immunofluorometric assay of free prostate-specific antigen with nanoparticle label technology. *Clin Chem* 2001;47:1269-78.
42. Taylor HE. Inductively coupled plasma-mass spectrometry: practices and techniques. San Diego: Academic Press, 2001:117-22.

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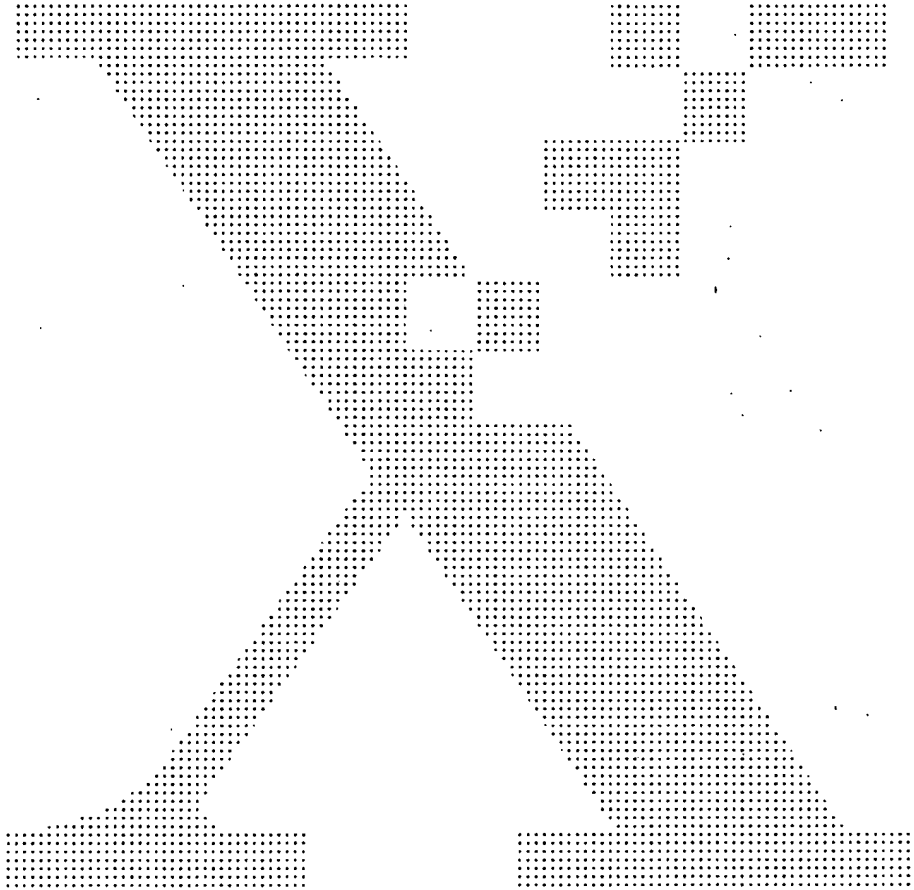


EXHIBIT V

TO DECLARATION OF SCOTT D. TURNER, PHD.

U.S. Patent Application Ser. No. 09/905,907

Simultaneous Determination of α -Fetoprotein and Free β -Human Chorionic Gonadotropin by Element-Tagged Immunoassay with Detection by Inductively Coupled Plasma Mass Spectrometry

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Background: An inductively coupled plasma mass spectrometry (ICP-MS)-based immunoassay has been proposed independently by Baranov et al. (*Anal Chem* 2002;74:1629–36) and our group, but the applicability of this method for multianalyte analysis in clinical samples has not been fully illustrated. We developed a dual-label immunoassay method for the simultaneous determination of α -fetoprotein (AFP) and free β -human chorionic gonadotropin (hCG β) in human serum.

Methods: Monoclonal antibodies immobilized on microtiter plates captured AFP and hCG β , which were detected by use of Eu³⁺-labeled anti-AFP and Sm³⁺-labeled anti-hCG β monoclonal antibodies. Eu³⁺ and Sm³⁺ were dissociated from the immunocomplex with HNO₃ solution (10 mL/L) and delivered by peristaltic pump to the ICP mass spectrometer.

Results: The measurable ranges of AFP and hCG β were 4.6–500 and 5.0–170 μ g/L, respectively, with detection limits of 1.2 and 1.7 μ g/L (3 SD above mean of zero calibrator), respectively. The intraassay imprecision (CV) for AFP was 8.3%, 4.0%, and 2.7% at 16.3, 86, and 354 μ g/L, respectively, and the interassay CV was 10%, 5.7%, and 3.5%. For hCG β , the intraassay CV was 5.4%, 6.4%, and 3.1%, respectively, at 10.5, 45.2, and 105 μ g/L, and the interassay CV was 7.2%, 8.0%, and 3.7%. Comparison with IRMAs for AFP and hCG β yielded correlation coefficients (r^2) of 0.97 and 0.95.

Conclusions: Two proteins can be measured simultaneously by immunoassays using two rare earth elemental tags (Eu³⁺ and Sm³⁺) and ICP-MS detection. The

multielement capability and the multiple potential elemental labels make ICP-MS attractive for multianalyte immunoassays. Implementation of ICP-MS-linked immunoassays may be relatively straightforward because the labeling and immunoreaction procedures have been well developed for clinical time-resolved immunofluorometric assays.

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Simultaneous multianalyte determinations are important for biological studies (1–5). Various multianalyte methods have been developed. Multianalyte immunoassays present several advantages, such as reduced analysis time, minimized repetitions of tedious procedures, and lower cost per test (6). Typically, these multianalyte immunoassay methods involve the use of more than one label. Although several kinds of labels, such as radiolabels or fluorescent, enzyme, or metal ion labels, have been used in the multianalyte immunoassays, the lanthanide chelate labels with time-resolved fluorescence detection are particularly attractive because of high sensitivity and other advantages (7–9). Recently, quantum dots such as CdSe have attracted attention because they can be prepared in a variety of sizes and their fluorescent properties are dependent on the size of the quantum dot (10,11). Having several quantum dots that all fluoresce at distinct wavelengths under identical excitation offers a dramatic advantage over fluorescence based on organic molecules, but the use of quantum dots in multiplexing are limited by the number of spectrally distinct species that can be prepared. Color-coded microspheres overcome many of the shortcomings of quantum dots because several hundred spectrally different types of beads can be prepared (12). Unfortunately, this labeling system is limited by the number of spectrally distinguished fluorophores, and its use for analyte quantification has encountered difficulties (13). Barcode labels circumvent the problems of fluorophores and quantum dots (14–16). However, the prepa-

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ration of barcode or bio-barcode labels is cumbersome, and the automated quantitative analysis still has some difficulties. In summary, the limitations to the reported approaches in the literatures for multianalyte determination include difficulty in finding a sufficient number of suitable labels that can be prepared easily and can be detected distinctly with similar sensitivities by a single detection technique.

Inductively coupled plasma mass spectrometry (ICP-MS),¹ a powerful technique for the simultaneous determination of multiple elements with minimal pretreatment of samples, offers excellent sensitivity for a wide range of elements. The combination of ICP-MS with immunoassays enables analysis of biological materials (17–23). Baranov et al. (18) described several novel ICP-MS-linked immunoassays that use both nanogold and lanthanide-tagged antibodies. Commonly used immunoaffinity separation techniques have been successfully coupled to ICP-MS to measure the concentrations of target proteins in complex biological samples. Parallel studies carried out in our laboratory used thyroid-stimulating hormone and thyroxine as model compounds to demonstrate the potential of ICP-MS as a detector for the Eu^{3+} that is attached to an immunoreagent (19,20). We also established a sandwich-type immunoreaction coupled to ICP-MS with colloidal gold nanoparticle-labeled antibody (21). The measurement of multiple proteins simultaneously by use of distinguishable element-tagged antibodies with ICP-MS has been illustrated by Quirun et al. (23), who used colloidal gold and Eu^{3+} as labels. As an excellent element-specific detection technique, the detection limits of ICP-MS for most elements range from parts per billion to parts per trillion, or even lower. Elements such as rare earths and noble metals, as well as some of the transition metals, have the highest sensitivity with detection limits down to 0.01–0.1 ng/L (24). Those elements, their stable isotopes, or the unique combination of them are candidates for labeling bioactive molecules, especially those that occur at naturally low concentrations in the body and environment. However, the value of ICP-MS-based immunoassays in clinical analysis has not been fully understood, and further research in this area is needed for the use of this methodology in practical applications.

α -Fetoprotein (AFP) and β -human chorionic gonadotropin (hCG β) are important tumor markers and also serve as aids in prenatal detection of fetal malformations (25–28). Numerous immunologic methods for determining AFP and hCG β concentrations have been described (29–35), but few methods other than time-resolved immunofluorometric assays and enzyme immunoassays can measure them simultaneously (28,36,37). In this report, we demonstrate the simultaneous measurement of AFP

and hCG β in clinical sample with ICP-MS as the end-point detection method. The commonly used elements in the present study, Eu^{3+} and Sm^{3+} , have detection limits down to 0.01 ng/L in ICP-MS with good biocompatibility and state-of-the-art bioconjugation methods. The proposed method shows the potentially powerful capability of ICP-MS-based multianalyte immunoassay for clinical use.

Materials and Methods

REAGENTS AND BUFFERS

AFP, hCG β , mouse monoclonal anti-AFP IgG, and mouse monoclonal anti-hCG β IgG for labeling or immobilization were from Scripps Laboratories. We used purified water (18 M Ω /cm) for all experiments. N' -[*p*-isothiocyanatobenzyl]-diethylene-triamine- N^1,N^2,N^3,N^3 -tetraacetate europium and N' -[*p*-isothiocyanatobenzyl]-diethylene-triamine- N^1,N^2,N^3,N^3 -tetraacetate-samarium were from Tianjing Radio Medical Institute. Microtiter strips were obtained from NUNC Co. Superpurity HNO_3 was obtained from Beijing Chemical Reagents Institute. Other reagents were of analytical grade.

The coating buffer was 100 mmol/L sodium carbonate buffer (pH 9.5) containing 9 g/L NaCl and 0.4 g/L NaN_3 . The blocking buffer was 50 mmol/L Tris-HCl (pH 7.0) containing 9 g/L NaCl, 0.4 g/L NaN_3 , and 10 g/L bovine serum albumin. The assay buffer was 50 mmol/L Tris-HCl (pH 7.8) containing 20 g/L bovine serum albumin, 0.4 g/L NaN_3 , 9 g/L NaCl, and 0.4 mL/L Tween 20. The wash buffer was 50 mmol/L Tris-HCl (pH 7.4) containing 0.4 g/L NaN_3 , 9 g/L NaCl, and 0.4 mL/L Tween 20.

INSTRUMENTATION

We used a Perkin-Elmer/Sciex Elan 6000 ICP mass spectrometer fitted with a cross-flow nebulizer. The instrument settings were optimized daily. Typically, nebulizer gas flow was 1.02 L/min, lens voltage was 8 V, and radio frequency (RF) power was 1150 W. Peak hopping mode was used in the experiment. The chromatographic separation system included a Model EP-1 Econo Pump and a Model EM-1 Econo UV monitor (Bio-Rad).

ANTIBODY LABELING

The anti-AFP and anti-hCG β monoclonal antibodies were labeled with Eu^{3+} and Sm^{3+} chelates, respectively. Briefly, 1 g/L antibody in 50 mmol/L sodium bicarbonate buffer (pH 9.5) was reacted with 0.35 mg of Eu^{3+} or Sm^{3+} chelate labeling reagent and incubated at 4 °C overnight. The Eu^{3+} - or Sm^{3+} -chelate-labeled antibody was purified on a Sephadex G-25 column (1.5 \times 25 cm) and eluted with 50 mmol/L Tris-HCl buffer (pH 7.0) containing 9 g/L NaCl and 0.5 g/L NaN_3 . The unlabeled Eu^{3+} concentration was determined by comparison with known Eu^{3+} standards. The concentration of Eu^{3+} -labeled anti-AFP monoclonal antibody was calculated as:

¹ Nonstandard abbreviations: ICP-MS, inductively coupled plasma mass spectrometry; AFP, α -fetoprotein; hCG β , β -human chorionic gonadotropin; and RF, radio frequency.

$$c = \frac{A_{280} - 0.008c_{Eu^{3+}}}{1.34}$$

where A_{280} is the absorbance of the Eu^{3+} -labeled anti-AFP monoclonal antibody at 280 nm, 1.34 is the absorbance of 1 g/L Eu^{3+} -labeled anti-AFP monoclonal antibody at 280 nm, and 0.008 is the absorbance of 1 μ mol/L Eu^{3+} chelate at 280 nm. The conjugation yield was obtained by calculating the ratio of the concentration of Eu^{3+} -labeled anti-AFP monoclonal antibody to the Eu^{3+} concentration in the solution. The conjugation yield of Sm^{3+} -labeled anti-hCG β monoclonal antibody was calculated similarly. One mole of anti-AFP monoclonal antibody was labeled with 7.1 moles of Eu^{3+} chelate, whereas 1 mole of anti-hCG β monoclonal antibody was labeled with 12.3 moles of Sm^{3+} chelate.

COATING OF MICROTITRATION WELLS

To immobilize the anti-AFP and anti-hCG β monoclonal antibodies on the well surfaces, we added 200 μ L of each to the wells at a concentration of 5 mg/L and allowed them to adsorb. The microtiter strips were washed twice with the wash solution, 300 μ L of blocking buffer was added to each well, and the strips were incubated overnight at 4 $^{\circ}$ C. After removal of the blocking buffer, the strips were stored at 4 $^{\circ}$ C in sealed bags until use.

ASSAY PROTOCOL

The typical sandwich format of a noncompetitive immunometric assay was used. The coated microtiter strip wells were washed twice with assay buffer, 25 μ L of calibrator or serum was pipetted into the well, and then 200 μ L of lanthanide-labeled anti-AFP and anti-hCG β antibodies (1 mg/L) was added to each microtiter well. Subsequently, the microtiter strip wells were incubated with continuous shaking at room temperature and then washed six times with wash buffer to separate the unreacted reagent from the microtiter strip well. We then added 200- μ L aliquots of HNO_3 solution (10 mL/L) to each well to dissociate the Eu^{3+} and Sm^{3+} ions from the labeled monoclonal antibodies. After 1 min of continuous shaking of the microtiter strips (40 rpm) at room temperature in the TS-1 shaking apparatus (Jiangsu), the solutions were introduced into the ICP-MS instrument by a peristaltic pump at a flow rate of 1 mL/min. The ^{153}Eu and ^{152}Sm intensities by ICP-MS were proportional to the concentrations of AFP and hCG β in the sample, respectively.

Two determinations were performed for each sample. Analytical results were obtained by calculating the mean values of two determinations of each sample. The correlation was calculated with a linear least-squares method, and agreement was tested by the Student *t*-test.

SAMPLE COLLECTION

Blood samples were collected from 20 patients at the Third Hospital of Peking University (Beijing, China), after the patients gave informed consent. After clotting, the

serum and packed cells were separated by centrifugation at 1000g. The serum samples were then stored at -20° C under nitrogen gas until they were assayed.

IRMA PROCEDURES

Serum AFP and hCG β were measured by the use of IRMAs (Beijing Chemclin Biotech Co., Ltd) according to the manufacturer's instructions. Briefly, 50 μ L of calibrator or serum sample was pipetted into a coated tube, after which 50 μ L of ^{125}I -labeled AFP or hCG β antibody was added to each coated tube. The microtiter strip wells were incubated for 2 h with continuous shaking at 37 $^{\circ}$ C and then washed five times with water to separate the unreacted reagent from the coated tubes. The radioactivity of the coated tube was measured to calculate the analyte concentration.

Results

OPTIMIZATION OF INSTRUMENT SETTINGS

The isotopes of highest abundance for both europium and samarium (^{153}Eu and ^{152}Sm) were chosen for this experiment. We used Eu^{3+} and Sm^{3+} standard solutions (5 μ g/L) to optimize key ICP-MS instrument settings, including RF power, nebulizer argon flow, and lens voltage. The dependence of ^{153}Eu and ^{152}Sm signal intensities on the argon nebulizer gas flow was studied at flow rates of 0.7–1.1 L/min. ^{153}Eu and ^{152}Sm behaved similarly, with a common peak at ~ 1 L/min (Fig. 1). The maximum intensity of ^{153}Eu was twice that of ^{152}Sm , reflecting the higher abundance of ^{153}Eu (52.2% for ^{153}Eu and 26.7% for ^{152}Sm). The chosen nebulizer gas flow (argon) for both ^{153}Eu and ^{152}Sm was 1.02 L/min for these studies. The RF power and lens voltage used in this experiment were also optimized; the optimum values were 1150 W and 8 V, respectively.

ASSAY CHARACTERISTICS

Calibration curve and detection limit. A typical calibration curve is shown in Fig. 2. The measurable range for AFP

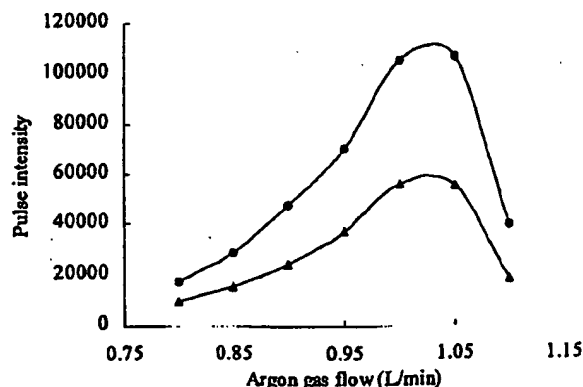


Fig. 1. Optimization of argon gas flow in ICP-MS. \bullet , ^{153}Eu ; Δ , ^{152}Sm . Sm^{3+} and Eu^{3+} concentration, 5 μ g/L; RF, 1250 W; lens voltage, 8 V.

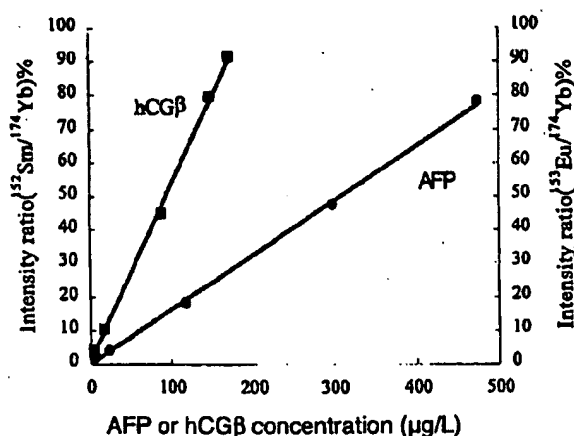


Fig. 2. Calibration curves for AFP (●) and hCGβ (■).

was 4.6–500 μg/L, and that for hCGβ was 5.0–170 μg/L. The regression equations for the calibration curves were: $I = 0.16c + 0.06$ for AFP ($r^2 = 0.9984$) and $I = 0.52c + 0.96$ for hCGβ ($R^2 = 0.9987$), where I is the relative intensity of the ICP-MS signal, and c is the concentration. The lower limits of detection, defined as the concentrations corresponding to a signal 3 SD above the mean of 12 replicates of the zero calibrator, were 1.2 μg/L for AFP and 1.7 μg/L for hCGβ.

Imprecision. The intraassay imprecision (CVs) were ~3–8% for AFP and ~3–5% for hCGβ (Table 1). The between-assay CVs for duplicate analyses over 6 days were 3.5–5.6% for AFP and 3.7–7.2% for hCGβ (Table 1).

Recovery. The recoveries of AFP (16.5, 84.6, and 213 μg/L) and hCGβ (6.8, 52.4, and 108 μg/L) added to samples (Table 2) were 90–104% and 88–110%, respectively.

Linearity. Three serum samples with high AFP and hCGβ concentrations were diluted with AFP- and hCGβ-free human serum. Measured concentrations were 90.8–119% of expected concentrations (Table 3).

Comparison with IRMA. Comparisons with IRMAs for AFP and hCGβ are shown in Figs. 3 and 4, respectively.

Table 1. Within- and between-assay imprecision for AFP and hCGβ.

Sample	Concentration, μg/L	Relative SD, %	
		Within-assay	Between-assay
AFP	16.3	8.3	9.6
	86.2	4.0	5.7
	354.0	2.7	3.5
hCGβ	10.5	5.4	7.2
	45.2	6.4	8.0
	105.0	3.1	3.7

* $n = 6$. Between-assay study used means of daily duplicates.

Table 2. Recoveries for AFP and hCGβ by the proposed ICP-MS-linked immunoassay.

Sample	Original, μg/L	Added, μg/L	Measured, μg/L	Recovery, %
AFP	16.5	48.0	66.5	104
	84.6	48.0	128	90
	213	48.0	260	98
hCGβ	6.8	25.0	28.9	88
	52.4	25.0	80	110
	108	25.0	132	96

The regression equations were: $y = 0.86x + 6.9$ μg/L ($r^2 = 0.97$) for AFP and $y = 1.00x - 0.64$ μg/L ($r^2 = 0.95$) for hCGβ. The standard deviation of the residuals was 4.1 μg/L for AFP ($n = 20$) and 3.3 μg/L for hCGβ ($n = 20$), respectively. The mean results were not significantly different (Student t -test).

STABILITIES OF LANTHANIDE-LABELED MONOCLONAL ANTIBODIES

We studied the stabilities of lanthanide-labeled mouse monoclonal anti-AFP IgG and mouse monoclonal anti-hCGβ IgG by storing each antibody (at 50 mg/L) at 4 °C for 1 week, 4 weeks, and 3 months before they were used in the assay. The calibration curves obtained were compared with the calibration curve obtained before the start of the storage experiment. No significant decrease in activity was observed.

Discussion

The most striking features of ICP-MS are its superior sensitivity for a wide range of elements and its ability to determine the isotope composition of a sample with pretreatment procedures that are less cumbersome than those for other MS techniques. However, the application of ICP-MS to biological fluid and tissue samples has been limited to the investigation of elements present in ultra-trace amounts (38, 39). ICP-MS analyses of biological samples need not be limited to analytes that include metal elements. The recent development of metal-tagged antibodies, which are designed for time-resolved immunofluorometric assays, makes possible the use of ICP-MS in immunoassays. Antigens of interest are reacted with their complementary metal-tagged antibodies. After separation

Table 3. Linearity as tested by dilutions of samples with AFP- and hCGβ-free human serum.

Sample	Original concentration, μg/L	Percentage of expected concentration				
		2-fold dilution	4-fold dilution	8-fold dilution	16-fold dilution	32-fold dilution
AFP	43.5	95	106	94	110	
	84.6	94	106	103	91	
	213	95	100	97	101	92
hCGβ	36.8	97	102	109		
	75.1	104	95	109	96	
	134	104	103	95	108	119

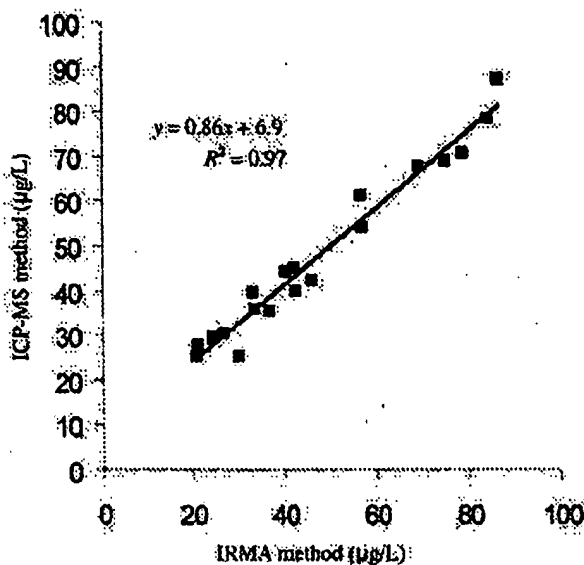


Fig. 3. Relationship between the proposed method and IRMA for AFP in human serum.

from nonreacting proteins, the elemental tags conjugated to the antibodies are measured by ICP-MS to determine the antigen concentrations in the samples (18–20). Because no special fluorescent properties are needed, the potential elemental labels for ICP-MS-linked immunoassay are not limited to the four lanthanides (Eu^{3+} , Sm^{3+} , Tb^{3+} , and Dy^{3+}).

The labels for ICP-MS-linked immunoassay should possess the properties of (a) having a highly sensitive response in ICP-MS and being free from spectral interference; (b) occurring at naturally low concentrations in the

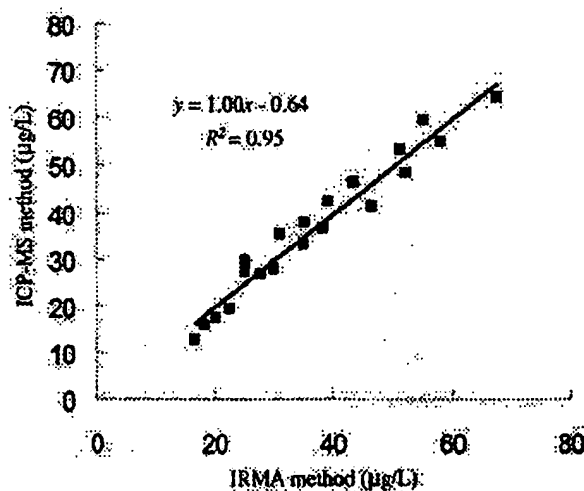


Fig. 4. Relationship between the proposed method and the IRMA for hCG β in human serum.

body and environment; and (c) having good biocompatibility and the ability to easily conjugate to biomolecules. On the basis of the above criteria, rare earth elements, such as europium, samarium, terbium, and dysprosium, can be considered ideal labels for ICP-MS-linked immunoassay because these elements offer good biocompatibility and are easily conjugated to biomolecules. Another advantage of the use of these elements as labels is that they offer high sensitivity for trace species in biological samples, which can satisfy the requirement of routine clinical analysis. In addition, a low background can be expected because these elements hardly exist in biological fluids or tissues. Those properties have been well demonstrated by many reports on time-resolved fluorescent immunoassays (7–9).

The other rare earth elements, including lutetium, yttrium, lanthanum, cesium, praseodymium, neodymium, gadolinium, holmium, erbium, thulium, ytterbium, and scandium, would also be potentially applied to ICP-MS-linked immunoassays because they could satisfy the first and second criteria mentioned above. Methods for their bioconjugation, however, have not been developed to the best of our knowledge. Noble metals, such as gold and ruthenium, would also be candidates for labels because ICP-MS can measure them at very low concentrations and they have been used in immunoassays with spectrometric and electrochemical detection. The other noble metals, such as silver, could also be determined sensitively by ICP-MS, but their biocompatibility is not satisfactory when conjugated to proteins. Some of the transition metals, such as iron, cobalt, and copper, can be measured with high sensitivity by ICP-MS, but these elements may also exist in biological fluids to some extent. Spectral interference may also be a problem, especially for the determination of iron-labeled biomolecules.

Quinn et al. (23) have discussed the simultaneous determination of two proteins with ICP-MS using nanogold clusters and Eu^{3+} as labels. One of the disadvantages of using nanogold clusters in this method is that gold has a high affinity for the surfaces of a typical ICP-MS sample introduction system, as pointed out by the authors. Another disadvantage is that the responses of nanogold clusters and Eu^{3+} in ICP-MS are not consistent, and two internal standards, iridium and holmium, must be used. Because lanthanides are a group of closely related elements having similar physical and chemical properties, a single or at most two internal standards are sufficient to control for instrumental drift for the determination of all of the lanthanides. Eu^{3+} and Sm^{3+} are used in the present study because, among all the elements in the periodic table, lanthanides are the most sensitively detected by ICP-MS, with detection limits down to 0.01 ng/L, and because they occur at very low concentrations in body fluids and the environment.

The detection limits of time-resolved immunofluoro-

metric assays for the simultaneous determination of AFP and hCG β with lanthanide chelates are 0.025 $\mu\text{g/L}$ for AFP and $\leq 0.2 \mu\text{g/L}$ for hCG β (36). The detection limits of the lanthanide-labeled ICP-MS-linked immunoassay were 1.2 $\mu\text{g/L}$ for AFP and 1.7 $\mu\text{g/L}$ for hCG β . The ratio of labeled lanthanide to corresponding antibody is an important factor affecting the sensitivity of detection of the ICP-MS-linked immunoassay because the ICP-MS signal intensity is linearly proportional to the number of atoms of the tags. It is possible that the detection limit of the present method can be improved by multiple labeling. In practice, however, improving the detection limit of an immunoassay by increasing the number of labels attached to an antibody has been limited because of the interference of the bulky labels in antibody-antigen binding and the increased fraction of nonspecific binding. In the present study, 1 mole of anti-AFP monoclonal antibody was labeled with 7.1 moles of Eu^{3+} , whereas the anti-hCG β monoclonal antibody was labeled with 12.3 moles of Sm^{3+} . As indicated in the *Results*, the maximum intensity of ^{153}Eu is twice that of ^{152}Sm at the same concentration because the natural abundance of ^{153}Eu is approximately twice that of ^{152}Sm , and we used those ratios only for the convenience of data processing. The nanoparticle-antibody bioconjugates provide a novel solution for this problem without compromising the binding affinity.

When measuring the element tags by MS, the signal enhancement is proportional to the number of atoms of the tag isotope. Because nanoparticles have significant numbers of atoms per conjugate, the increase in sensitivity is obvious. In a previous work, we developed an immunoassay by coupling a sandwich-type immunoreaction to ICP-MS with colloidal gold nanoparticles as labels on goat anti-rabbit IgG (21). In their study, another group showed that different immunoassay methods, such as centrifugal filtration, protein A affinity, and size-exclusion gel filtration, can be linked with ICP-MS immunoassays using nanogold cluster labels (18). However, the use of colloidal gold or nanogold clusters as labels is limited by constraints of specificity and immunoreactivity, and continuing efforts to alleviate the nonspecific binding are required for further improvements in sensitivity.

An alternative method is to use lanthanide-dye-conjugated polystyrene nanoparticles as labels; these types of nanoparticles were used recently in a time-resolved immunofluorometric assay and had the advantages of enhanced monovalent binding affinity and a high specific activity for the nanoparticle-antibody bioconjugate (40, 41). The nanoparticle, 107 nm in diameter, contained >30 000 europium atoms and could be used in ICP-MS-linked immunoassays to improve the detection limit without the problems of diminished activity and specificity. It can therefore be inferred that element-labeled ICP-MS immunoassays can provide sensitivities comparable to those for time-resolved immunofluorometric assays with the potential for use in multianalyte determinations incorporating large numbers of tags.

ICP-MS-linked immunoassays have many advantages over conventional time-resolved immunofluorometric assays (18). ICP-MS-linked immunoassays do not require addition of enhancement solution, as time-resolved immunofluorometric assays do, because the lanthanides themselves are detected directly. The dissociation of lanthanides from antigen-antibody complexes by acidification enables long-term storage before analysis, simplifying assay protocols. The inherent ability of ICP-MS to remain free from interference by organic or inorganic species makes the operational procedure easier. The immunoassay steps are the same as for time-resolved fluorescence except for the detection step. After immunoreaction, enhancement solution is added to the microtiter strip well, which is shaken for 5 min to allow the formation of fluorescent complex for time-resolved fluorescence detection, whereas HNO_3 solution (10 mL/L) is added to dissociate Eu^{3+} and Sm^{3+} ions from the labeled monoclonal antibodies for ICP-MS detection. Because the immunoreaction steps of the two methods are identical, their analytical throughput is the key for comparing their analytical ability. The throughput of time-resolved fluorescence is 1–2 s/sample with automated instruments. In the present study, the throughput was ~ 1 min/sample for two elements. One of the reasons for the lower throughput of the ICP-MS detection immunoassay is that the sampling system was not designed specifically for immunoassays. The advantage of ICP-MS is its ability to analyze multiple analytes, not its analysis speed, compared with time-resolved fluorescence. Because the antibody preparation and immunoreaction steps in the ICP-MS-linked immunoassay are the same as those in time-resolved immunofluorometric assays, which are familiar to clinical analysts, this method can be easily accepted and used by clinical chemists.

One of the promising applications of element-labeled immunoassay combined with ICP-MS detection is in the development of SI-traceable primary methods for interlaboratory comparisons and quality assurance by so-called isotope dilution. Isotope dilution is a calibration technique based on altering the natural relative abundance of two stable isotopes by adding a known amount of an enriched isotope of the analyte to the sample (42). Measurement of this altered ratio allows very accurate quantification because the added enriched isotope acts analogously to an ideal internal standard. The uncertainty of the exact isotopic ratio, the amount of the enriched analyte added, and the weight or volume of the sample are the only sources of error that can affect the results. Therefore, isotope-dilution analysis provides a definitive technique and absolute quantification with excellent precision and accuracy compared with other analytical methods. The possibility of using an isotope dilution in the present method has been under investigation in our laboratory.

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References

- MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science* 2000;289:1760-3.
- Chan WCW, Maxwell DJ, Gao XH, Bailey RE, Han MY, Nie SM. Luminescent quantum dots for multiplexed biological detection and imaging. *Curr Opin Biotechnol* 2002;13:40-6.
- Moreno-Bondi MC, Alarie JP, Vo-Dinh T. Multi-analyte analysis system using an antibody-based biochip. *Anal Bioanal Chem* 2003;375:120-4.
- Rowe CA, Scruggs SB, Feldstein MJ, Golden JP, Ligler FS. Array biosensor for simultaneous identification of bacterial, viral, and protein analytes. *Anal Chem* 1999;71:433-9.
- Delehanty JB, Ligler FS. A microarray-immunoassay for simultaneous detection of proteins and bacteria. *Anal Chem* 2002;74:5681-7.
- Brecht A, Abuknesha R. Multianalyte immunoassays application to environmental analysis. *Trac-Trend Anal Chem* 1995;14:361-71.
- Wu FB, Han SQ, Xu T, He YF. Sensitive time-resolved fluorimetric immunoassay for simultaneous detection of serum thyroid-stimulating hormone and total thyroxine with Eu and Sm as labels. *Anal Biochem* 2003;314:87-96.
- Bookout JT, Joaquim TR, Magin KM, Rogan GJ, Lirette RP. Development of a dual-label time-resolved fluorimetric immunoassay for the simultaneous detection of two recombinant proteins in potato. *J Agric Food Chem* 2000;48:5868-73.
- Eriksson S, Vehniainen M, Jansen T, Meretoja V, Saviranta P, Pettersson K, et al. Dual-label time-resolved immunofluorometric assay of free and total prostate-specific antigen based on recombinant Fab fragments. *Clin Chem* 2000;46:658-66.
- Bruchez M Jr, Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor nanocrystals as fluorescent biological labels. *Science* 1998;281:2013-5.
- Chan WCW, Nie SM. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. *Science* 1998;281:2016-8.
- Han MY, Gao X, Su JZ, Nie SM. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat Biotechnol* 2001;19:631-5.
- Reiss BD, Freeman RG, Walton ID, Norton SM, Smith PC, Stonas WG, et al. Electrochemical synthesis and optical readout of striped metal rods with submicron features. *J Electroanal Chem* 2002;522:95-103.
- Nicewarner-Pena SR, Freeman RG, Reiss BD, He L, Pena DJ, Walton ID, et al. Submicrometer metallic barcodes. *Science* 2001;294:137-41.
- Walton ID, Norton SM, Balasingham A, He L, Ovisio DF, Gupta D, et al. Particles for multiplexed analysis in solution: Detection and identification of striped metallic particles using optical microscopy. *Anal Chem* 2002;74:2240-7.
- Nam JM, Thaxton CS, Mirkin CA. Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science* 2003;301:1884-6.
- Deng AP, Liu HT, Jiang SJ, Huang HJ, Ong CW. Reaction cell inductively coupled plasma mass spectrometry-based immunoassay using ferrocene tethered hydroxysuccinimide ester as label for the determination of 2,4-dichlorophenoxyacetic acid. *Anal Chim Acta* 2002;472:55-61.
- Baranov VI, Quinn Z, Bandura DR, Tanner SD. A sensitive and quantitative element-tagged immunoassay with ICPMS detection. *Anal Chem* 2002;74:1629-36.
- Zhang C, Wu FB, Zhang XR. ICP-MS-based competitive immunoassay for the determination of total thyroxine in human serum. *J Anal At Spectrom* 2002;17:1304-7.
- Zhang C, Wu FB, Zhang YY, Wang X, Zhang XR. A novel combination of immunoreaction and ICP-MS as a hyphenated technique for the determination of thyroid-stimulating hormone (TSH) in human serum. *J Anal At Spectrom* 2001;16:1393-6.
- Zhang C, Zhang ZY, Yu BB, Shi JJ, Zhang XR. Application of the biological conjugate between antibody and colloid Au nanoparticles as analyte to inductively coupled plasma mass spectrometry. *Anal Chem* 2002;74:96-9.
- Baranov VI, Quinn Z, Bandura DR, Tanner SD. The potential for elemental analysis in biotechnology. *J Anal At Spectrom* 2002;17:1148-52.
- Quinn ZA, Baranov VI, Tanner SD, Wrona JL. Simultaneous determination of proteins using an element-tagged immunoassay coupled with ICP-MS detection. *J Anal At Spectrom* 2002;17:892-6.
- Horlick G, Montaser A. Analytical characteristics of ICPMS. In: Montaser A, ed. *Inductively coupled plasma-mass spectrometry*. New York: Wiley-VCH, 1998:506-7.
- Berry E, Aitken DA, Crossley JA, Macri JN, Connor JM. Analysis of maternal serum α -fetoprotein and free β -human chorionic gonadotropin in the first trimester—implications for down-syndrome screening. *Prenat Diagn* 1995;15:555-65.
- Gregory JJ, Finlay JL. α -Fetoprotein and β -human chorionic gonadotropin—their clinical significance as tumour markers. *Drugs* 1999;57:463-7.
- Norgaardpedersen B, Alfthan H, Arends J, Hogdall CK, Larsen SO, Stenman UH, et al. A new simple and rapid dual assay for α -AFP and free β -hCG in screening for down-syndrome. *Clin Genet* 1994;45:1-4.
- Ito K, Oda M, Tsuji A, Maeda M. Simultaneous determination of α -fetoprotein, human chorionic gonadotropin and estradiol in serum of pregnant women by time-resolved fluorimetric immunoassay. *J Pharm Biomed Anal* 1999;20:169-78.
- Perkins S, Collins S, Kerten E, McKeon K, Mossner E, Saul R. An automated electrochemiluminescence immunoassay for the determination of α -1-fetoprotein (AFP). *Clin Chem* 1995;41:S67-8.
- Chu X, Lin ZH, Shen GL, Yu RQ. Studies on the piezoelectric immunosensor for the determination of α -fetoprotein (AFP). *Chem J Chin Univ* 1996;17:870-3.
- Hall LO, Haehner B, Swann S, Feliciano JA, Feliciano D, Miller D, et al. Development of a quantitative method for the determination of total β -hCG from whole blood for the Stratus[®] CSSTAT fluorimetric analyzer [Abstract]. *Clin Chem* 2000;46(Suppl 6):A11.
- Santandreu M, Alegret S, Fabregas E. Determination of β -HCG using amperometric immunosensors based on a conducting immunocomposite. *Anal Chim Acta* 1999;396:181-8.
- Gunupudi SR, Cheung H, Blem J, Bethell D. Quantitative determination of HCG using Medix Biotech's contrast HCG urine/serum test [Abstract]. *Clin Chem* 1996;42(Suppl 6):S130.
- Mack M, Oed M, Amtmann R, Schlett R. Liaison[®] hCG—an automated chemiluminescent immunoassay for the determination of human chorionic gonadotropin [Abstract]. *Clin Chem* 1997;43(Suppl 6):S174.
- Yamagata Y, Katoh H, Nakamura K, Tanaka T, Satomura S, Matsuura S. Determination of α -fetoprotein concentration based on liquid-phase binding assay using anion exchange chromatography and sulfated peptide introduced antibody. *J Immunol Methods* 1998;212:161-8.
- Pettersson K, Alfthan H, Stenman UH, Turpeinen U, Suonpaa M, Soderholm J, et al. Simultaneous assay of α -fetoprotein and free β subunit of human chorionic gonadotropin by dual-label time-resolved immunofluorometric assay. *Clin Chem* 1993;39:2084-9.
- Macri JN, Spencer K, Anderson R. Dual analyte immunoassays

- new approach to neural tube defect and Down's syndrome screening. *Ann Clin Biochem* 1992;29:390-6.
38. Sanz-Medel A, Montes-Bayon M, Sanchez MLF. Trace element speciation by ICP-MS in large biomolecules and its potential for proteomics. *Anal Bioanal Chem* 2003;377:236-47.
39. Patriarca M. The contribution of inductively coupled plasma mass spectrometry to biomedical research. *Microchem J* 1996;54:262-71.
40. Hama H, Soukka T, Lonnberg S, Paukkunen J, Tarkkinen P, Lovgren T. Zeptomole detection sensitivity of prostate-specific antigen in a rapid microtitre plate assay using time-resolved fluorescence. *Luminescence* 2000;15:351-5.
41. Soukka T, Paukkunen J, Hama H, Lonnberg S, Lindroos H, Lovgren T. Supersensitive time-resolved immunofluorometric assay of free prostate-specific antigen with nanoparticle label technology. *Clin Chem* 2001;47:1269-78.
42. Taylor HE. Inductively coupled plasma-mass spectrometry: practices and techniques. San Diego: Academic Press, 2001:117-22.